Exhibit B

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Induction of a Common Pathway of Apoptosis by Staurosporine

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The present observations show that staurosporine can rapidly trigger both the morphological changes and intranucleosomal DNA fragmentation typical of apoptosis. This occurred in a number of cell lines from various origins regardless of the state of differentiation and cell cycle phase, suggesting the presence of a common inducible suicide pathway. The broad apoptotic activity of staurosporine appears to be unique among other protein kinase or phosphatase inhibitors we tested. Results obtained in a cell-free assay suggest that cytoplasmic proteins directly modulated by staurosporine may be involved in a ubiquitous signal for the induction of DNA fragmentation and apoptosis. © 1994 Academic Press, Inc.

INTRODUCTION

Staurosporine, an alkaloid isolated from Streptomyces cultures [1] is a potent protein kinase inhibitor with a broad spectrum of activity [2–4]. Recently, staurosporine has been shown to inhibit cell cycle progression in a variety of cell lines [5–7], to enhance differentiation of human promyelocytic leukemia cells [8, 9], to inhibit tumor cell invasion [10], to induce the morphological changes typical of apoptosis in MOLT-4 cells [11] and human fibroblasts [12], and to induce DNA fragmentation in HL-60 cells [13].

Programmed cell death (apoptosis) is a fundamental process in embryogenesis, immune system maturation, and tissue homeostasis. Its deregulation may have important implications for immune system disorders and carcinogenesis. New findings suggest that blocking programmed cell death may be as important in cancer development as increasing cell proliferation. Conversely, tumor cells could be selectively eliminated by artificially triggering their death or suicide through apoptosis [for review, see 14, 15]. Many studies indicate that apoptosis can be modulated by factors that are associated with intracellular signaling pathways, suggesting the presence of multiple pathways of apoptosis [13–17]. Until now, however, the induction of apoptosis *in vitro* has been limited to certain cell models, suggesting that these cells are at least partially programmed for apoptosis [17]. Although those systems provide important models for studying programmed cell death, the finding of a common stimulus may prove to be useful in understanding the biochemical chain of events involved in apoptosis. In the present study we show that staurosporine induces apoptosis in a variety of human tumor cell lines, suggesting that protein phosphorylation plays a central role in triggering apoptosis.

MATERIALS AND METHODS

Chemicals. Staurosporine, KT-5720, KT-5823, and calphostin C were obtained from Kamiya Biomedical Company (Thousand Oaks, CA) and okadaic acid from UBI (Lake Placid, NY). Aphidicolin, cycloheximide, calmidazolium, 3-aminobenzamide, spermine, and 12-*O*-tetradecanoylphorbol 13-acetate (TPA), were purchased from Sigma. Radiolabeled precursors were purchased from New England Nuclear (Boston, MA). All other chemicals were of reagent grade and purchased either from Sigma or other local sources.

Cell culture and DNA labeling. Human promyelocytic leukemia HL-60 cells and follicular lymphoma SUDHL16 cells were obtained from Dr. Breitman and Dr. Bang (NCI, Bethesda, MD), respectively; all other cell lines were from American Type Cell Culture (Rockville, MD). HL-60, CA46, and SUDHL16 cells were grown in suspension culture in RPMI 1640 medium [Advanced Biotechnologies Incorporated (ABI), Columbia, MD] and HT29, SCL209, and DC3F cells were grown in Eagle's minimum essential medium (ABI). Cells were cultured at 37°C in the presence of 5% CO_2, and media were supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), 2 m*M* glutamine, 100 U penicillin/ml, and 100 μg streptomycin/ml (ABI). HL-60 cells were induced to differentiate along the monocytic/macrophagic pathway by treatment with 10 n*M* TPA for 24 h and only the adherent cells were used as differentiated. Synchronized CA46 cells were obtained following a double-aphidicolin block strategy and cell cycle analysis was performed using a fluorescence-activated cell analyzer (Becton–Dickinson). Data were interpreted using the SOBR model analysis program. For measurement of DNA fragmentation, exponentially growing cells were pre-labeled with [^{14}C]thymidine (0.02 μCi/ml) for 24 h and then chased in isotope-free medium overnight prior to drug treatment.

Electron microscopy. Control and treated cells (1×10^7 cells) were pelleted by centrifugation and washed twice with PBS. Fixation was performed in Millonig's sodium phosphate (pH 7.4, 292 mOsm) containing 2.5% glutaraldehyde, staining with 2% uranyl acetate, and dehydration with several ethanol treatments. Sections (500 to 700 Å thick) were mounted on copper grids and stained in lead citrate. Samples were examined (JFE Enterprises, Brookeville, MD) by transmission electron microscopy using a Ziess Em10 CA microscope.

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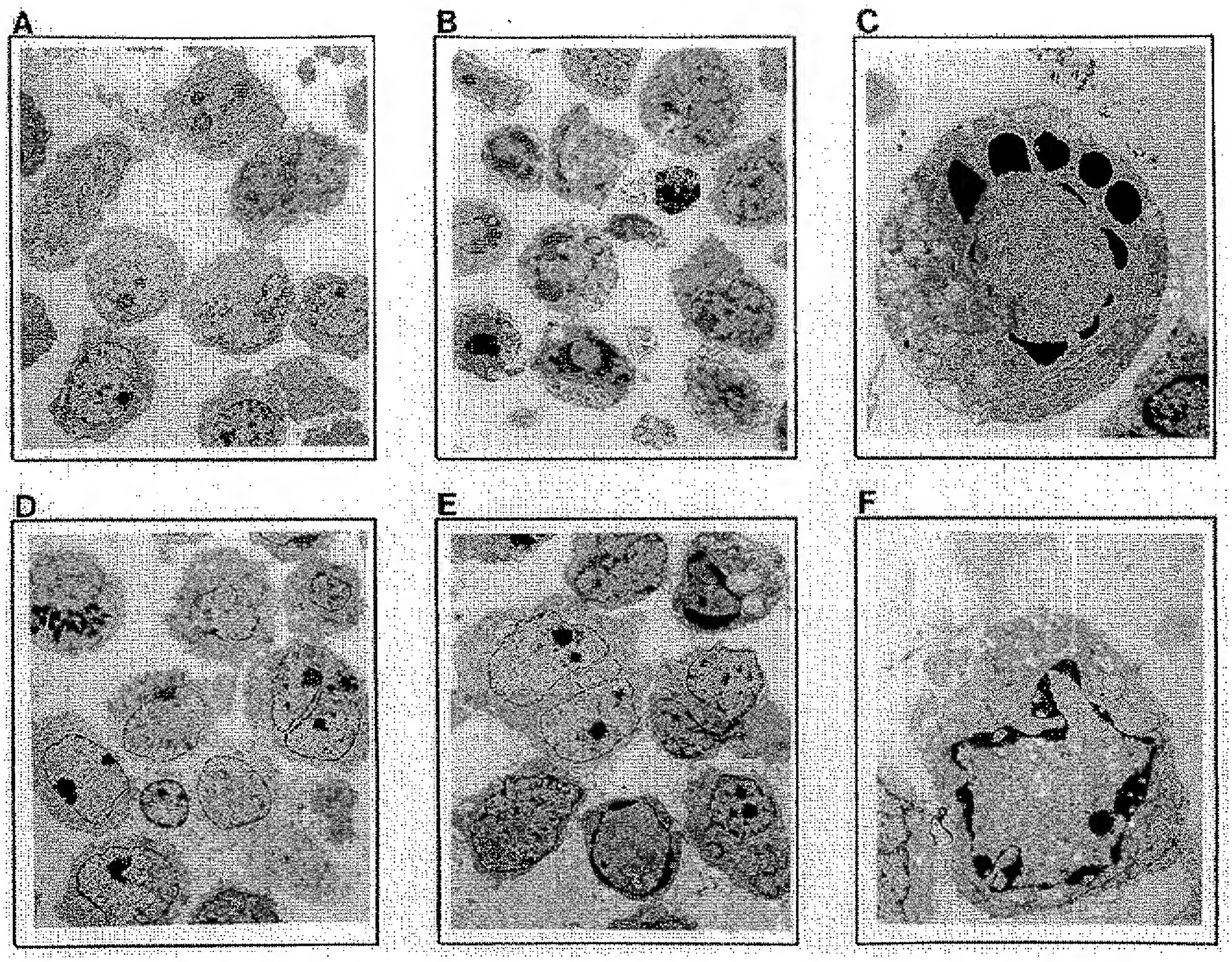

FIG. 1. Morphological appearance of human promyelocytic leukemia (HL-60) and Burkitt lymphoma (CA46) cells treated with staurosporine (1 μM for 3 h). Cells were processed for electron microscopy as described under Materials and Methods and samples were then examined by transmission electron microscopy using a Zeiss EM10 CA microscope. HL-60 cells are shown in the upper panels: (A) untreated cells (magnification 6225), (B and C) staurosporine-treated cells (magnification 4980 and 19,920, respectively). CA46 cells are shown in the lower panels: (D) untreated cells (magnification 4980), (E and F) staurosporine-treated cells (magnification 6225 and 15,687, respectively).

Determination of DNA secondary fragmentation by filter binding assay. DNA fragmentation involved in apoptosis was measured by the filter binding assay as described previously under nondeproteinizing conditions [13, 18, 19]. Approximately 0.5×10^6 prelabeled cells with [2-^{14}C]thymidine were directly loaded onto protein adsorbing filters [vinyl/acrylic copolymers filters, Metricel membrane, 0.8-μm pore size, 25-mm diameter (Gelman Sciences Incorporated, Arbor, MI)] at specified times after drug treatment. Cells were then washed with an additional 5 ml of ice-cold Hanks' balanced salt solution. As soon as the washing solution had dripped through by gravity, lysis was performed with 5 ml of LS-10 solution (0.2% sodium sarkosyl–2 M NaCl–0.04 M EDTA pH 10.0). After the lysis had dripped through by gravity, it was washed from the filter with 5 ml of 0.02 M EDTA (pH 10.0). Filters were then processed for liquid scintillation counting. DNA fragmentation was determined as the fraction of ^{14}C-labeled DNA in the lysis fraction + EDTA wash relative to total intracellular ^{14}C-labeled DNA. Results are expressed as the percentage of DNA fragmentation in treated cells compared to DNA fragmentation in control untreated cells (background) using the formula $(F - F_0/1 - F_0) \times 100$, where F and F_0 represent DNA fragmentation in treated and control cells, respectively.

Analysis of DNA fragmentation by agarose gel electrophoresis. At specified times following drug treatment, cellular DNA was extracted by a salting-out procedure (Stratagene, La Jolla, CA). Electrophoresis was then performed in 1.2% agarose gel in Tris–borate buffer (pH 8.0) at 20 V for approximately 14 h. After electrophoresis, DNA was visualized by ethidium bromide staining.

Reconstituted cell-free system. Cytoplasmic and nuclear fractions were prepared as follows: HL-60 cells were spun down and washed four times by centrifugation/resuspension in 10 ml ice-cold PBS (without Ca^{2+} and Mg^{2+}) and incubated on ice for 10 min at a density of 1.0×10^7 cells/ml in a lysis buffer containing 150 mM NaCl, 1 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 0.15 U/ml

aprotinin, 1.0 m*M* Na_3VO_4, 5 m*M* Hepes (pH 7.4), 10% glycerol, and 0.3% Triton X-100. Lysates were then centrifuged (2000*g* for 10 min at 4°C) and supernatants were collected as cytoplasmic fractions. Pellets (nuclei fractions) were then washed twice by centrifugation/resuspension in the lysis buffer without Triton. Cytoplasm from untreated or treated cells was then incubated with isolated nuclei from labeled untreated cells at 30°C for the indicated times. DNA fragmentation was then measured by filter binding assay and visualized by an ethidium bromide-stained agarose gel after electrophoresis [19].

RESULTS

Morphological changes induced by staurosporine. Staurosporine-treated human promyelocytic leukemia (HL-60) and Burkitt lymphoma (CA46) cells undergo morphological changes in cell structure typical of apoptosis [11] (Fig. 1). The chromatin becomes condensed at the periphery of the nuclei and forms dense micronuclear bodies (see Fig. 1C). The cell volume is reduced and the plasma membrane remains well defined, in agreement with trypan blue exclusion. Some organelles such as mitochondria remain intact during the early stages, while others including the endoplasmic reticulum and golgi apparatus appear dilated.

DNA fragmentation induced by staurosporine. Another key event of apoptosis is the cleavage of DNA into oligonucleosomal-sized fragments [16]. We adopted our previously developed filter assay to evaluate the kinetics of DNA fragmentation [18] in cell lines of diverse origins (Figs. 2 and 3). This assay allows accurate quantitation of non-protein-linked double-stranded DNA fragments directly from cell culture [18]. As expected, both HL-60 and CA46 cells demonstrated extensive DNA fragmentation about the time peripheral nuclear chromatin condensation was observed (compare Figs. 1–3). Figure 2 shows that the effects of staurosporine were time- and concentration-dependent with detectable effects at 0.1 μM staurosporine in HL-60 cells. Trypan blue remained excluded at the lowest staurosporine concentrations and earliest time, consistent with plasma membrane integrity. Trypan blue positivity at 10 μM probably corresponds to necrosis secondary to apoptosis (Fig. 2). Interestingly, we found that the other cell lines tested, which were generally regarded as refractory to cell death processing by an apoptotic pathway [18, 19], were sensitive to staurosporine-induced DNA fragmentation into oligonucleosomes (Fig. 3). Most striking was the induction of apoptosis in terminally differentiated HL-60 cells and in colon carcinoma HT-29 cells. SUDHL6 follicular lymphoma cells, which overexpress bcl2 oncogene [20] and are generally believed to be protected from apoptosis, were also sensitive to staurosporine. All cell lines treated exhibited intact plasma membranes (as determined by trypan blue exclusion) and marked volume shrinkage over the same time course as DNA fragmentation.

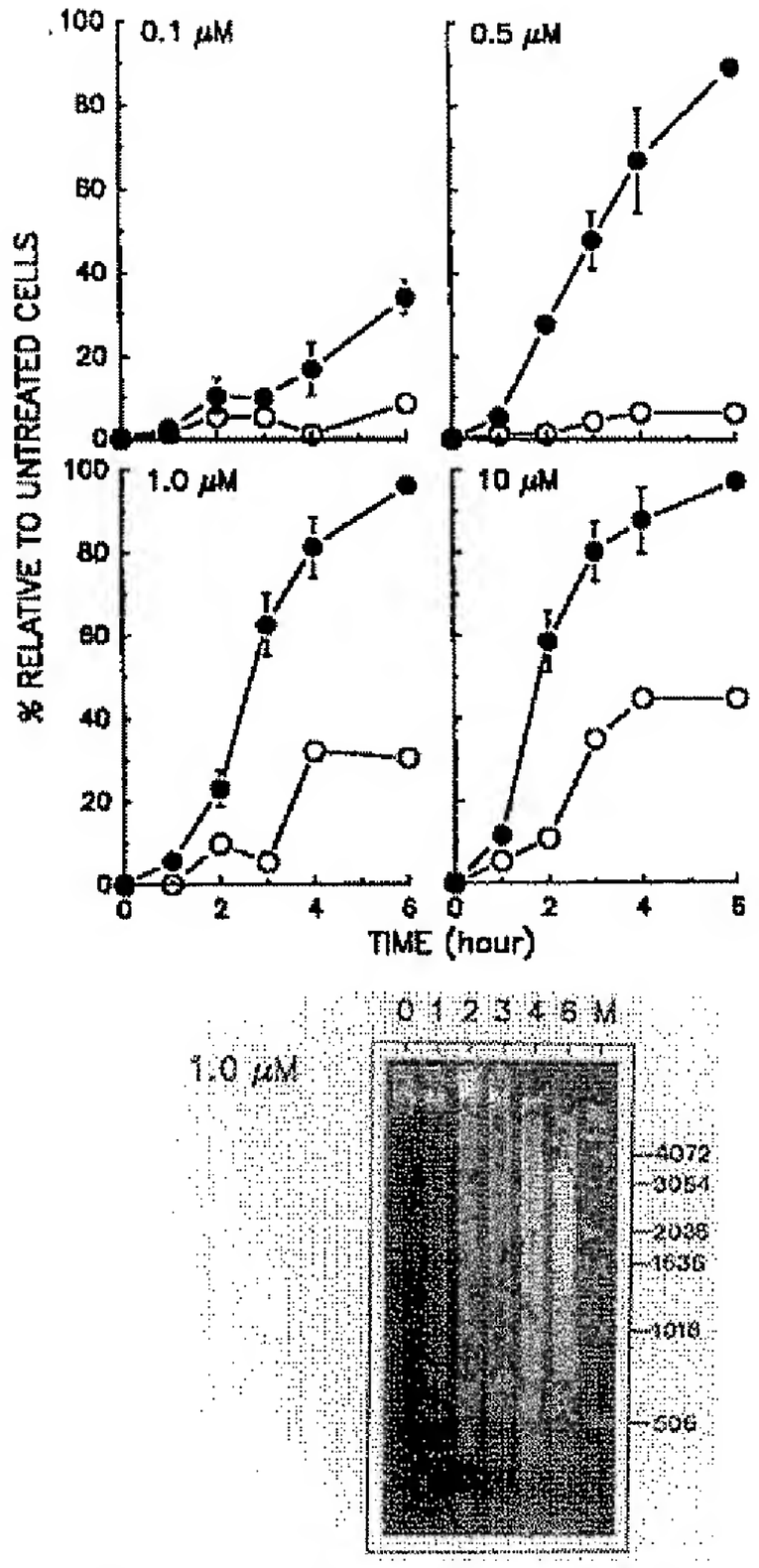

FIG. 2. Staurosporine-induced DNA fragmentation in HL-60 cells. (Upper panels) At indicated times following staurosporine treatment at 0.1, 0.5, 1.0, and 10 μM, DNA fragmentation (closed circles) was determined by filter binding assays and membrane impermeability (open circles) by trypan blue exclusion assays. Values shown are means ± SD of three independent experiments. (Lower panel) DNA fragmentation visualized as oligonucleosome-sized fragments in ethidium bromide-stained agarose gels. Treatments of 1.0 μM staurosporine were for the indicated times (number above each lane). Sizes of molecular weight markers (M) are indicated to the right.

Effect of cell cycle distribution upon staurosporine-induced apoptosis. Staurosporine has been shown to arrest cells in G1 and G2 of the cell cycle [5–7]. To explore whether staurosporine-induced apoptosis was specific to cells in G1 and/or G2 of the cell cycle we assayed for apoptosis in synchronized cultures (Fig. 4). CA46 cells were synchronized at the G1/S boundary using a

CELL LINES	% OF DNA FRAGMENTATION 2 HOURS	4 HOURS	8 HOURS
HL60 / PROMYELOCYTIC LEUKEMIA^{Hu}	23.2 ± 6.3	81.3 ± 10.3	96.4 ± 1.7
HL60^{TPA} / MACROPHAGIC DIFFERENTIATION^{Hu}	17.3 ± 0.7	58.5 ± 5.8	96.5 ± 0.7
CA46 / BURKITT'S LYMPHOMA^{Hu}	9.0 ± 3.3	22.5 ± 6.2	41.8 ± 10.1
SUDHL6 / FOLLICULAR LYMPHOMA^{Hu}	3.9 ± 1.6	12.9 ± 0.9	24.2 ± 9.1
HT29 / COLON ADENOCARCINOMA^{Hu}	0.0 ± 0.0	6.6 ± 4.2	25.6 ± 6.3
SCL209 / SMALL CELL LUNG CARCINOMA^{Hu}	ND	ND	56.9 ± 9.8
DC3F / LUNG FIBROBLAST^{Ch}	1.7 ± 1.7	19.5 ± 7.9	52.8 ± 9.8

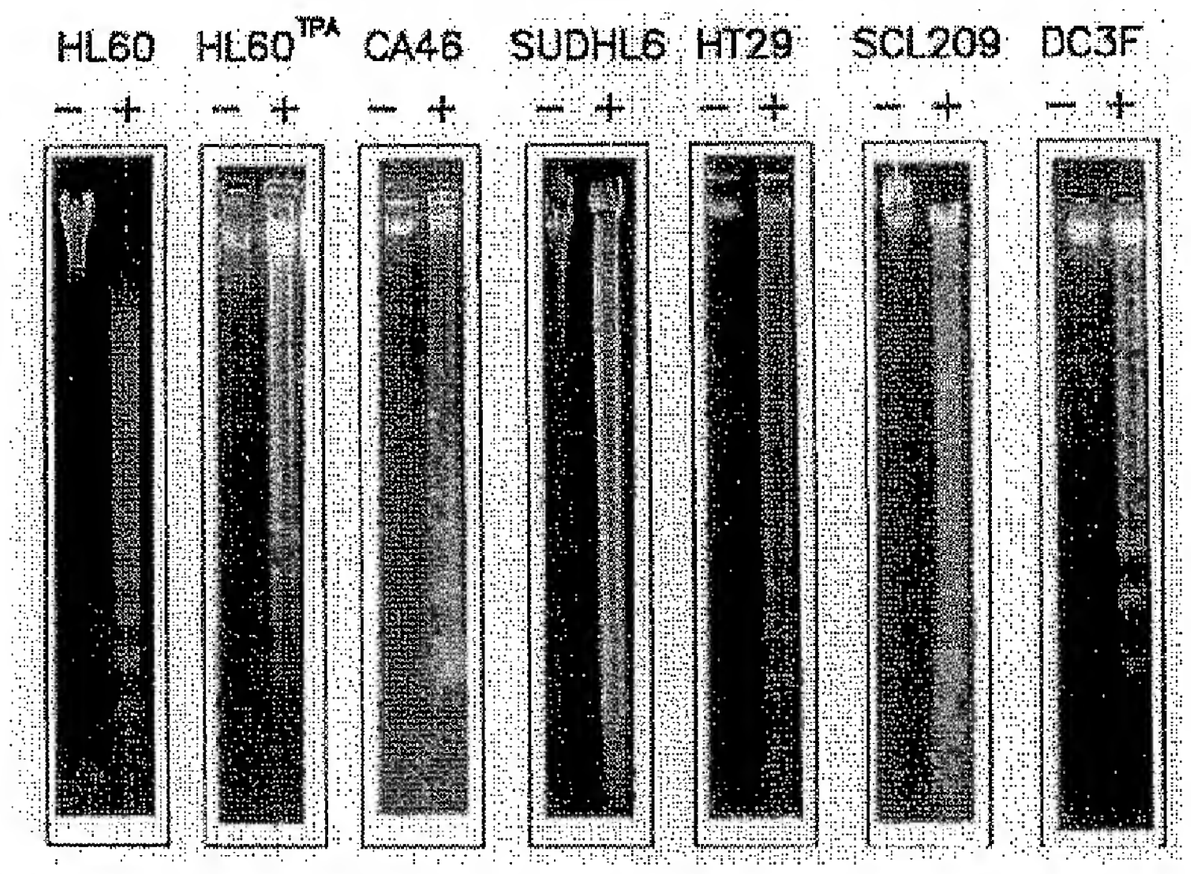

FIG. 3. Staurosporine-induced DNA fragmentation in a variety of cell lines. (Upper panel) DNA fragmentation measured by filter binding assays. Staurosporine treatments were for 2, 4, and 8 h at 1 μM. Values shown are means ± SD of three independent experiments. Superscripts Hu and Ch indicate human and Chinese hamster cell lines, respectively. (Lower panels) DNA fragmentation visualized as oligonucleosome-sized fragments in ethidium bromide-stained agarose gels. Treatments of staurosporine (1 μM) were for 4 h in the case of HL-60^{TPA} and CA46 cells and for 8 h in the other cells lines. (−) and (+) indicate DNA extracted from untreated control and staurosporine-treated cells, respectively.

double-aphidicolin block strategy and then released into fresh medium. Cell cycle progression was monitored by flow cytometry. Staurosporine was added at 2.5-h intervals following release from aphidicolin block and apoptosis was assayed by DNA fragmentation. Following release from aphidicolin block, CA46 cells progressed synchronously across the cell cycle, allowing the actions of staurosporine to be tested throughout a complete cell cycle. We were able to obtain a relatively pure population of cells in S, G2/M, or G1 of the cell cycle at 2.5, 5, and 10 h, respectively. Staurosporine induced a dose-dependent increase in DNA fragmentation across the cell cycle. Furthermore, cells progressing into G1 of the next cell cycle appeared to be slightly more sensitive to apoptosis induced by 1 μM staurosporine compared to cells in S or G2 phases (Fig. 4).

Effects of known modulators of apoptosis upon staurosporine-induced apoptosis. Protein synthesis was not required for the induction of apoptosis since cycloheximide, a protein synthesis blocker, did not inhibit the effect of staurosporine in CA46 cells. Similarly, commonly used modulators in other types of apoptosis such as the phorbol ester TPA [21], the calmodulin inhibitor calmidazolium [22], the polyamine spermine [13, 23], the poly(ADP-ribose) synthetase inhibitor 3-aminobenzamide [13, 24], or zinc [24] were ineffective in modulat-

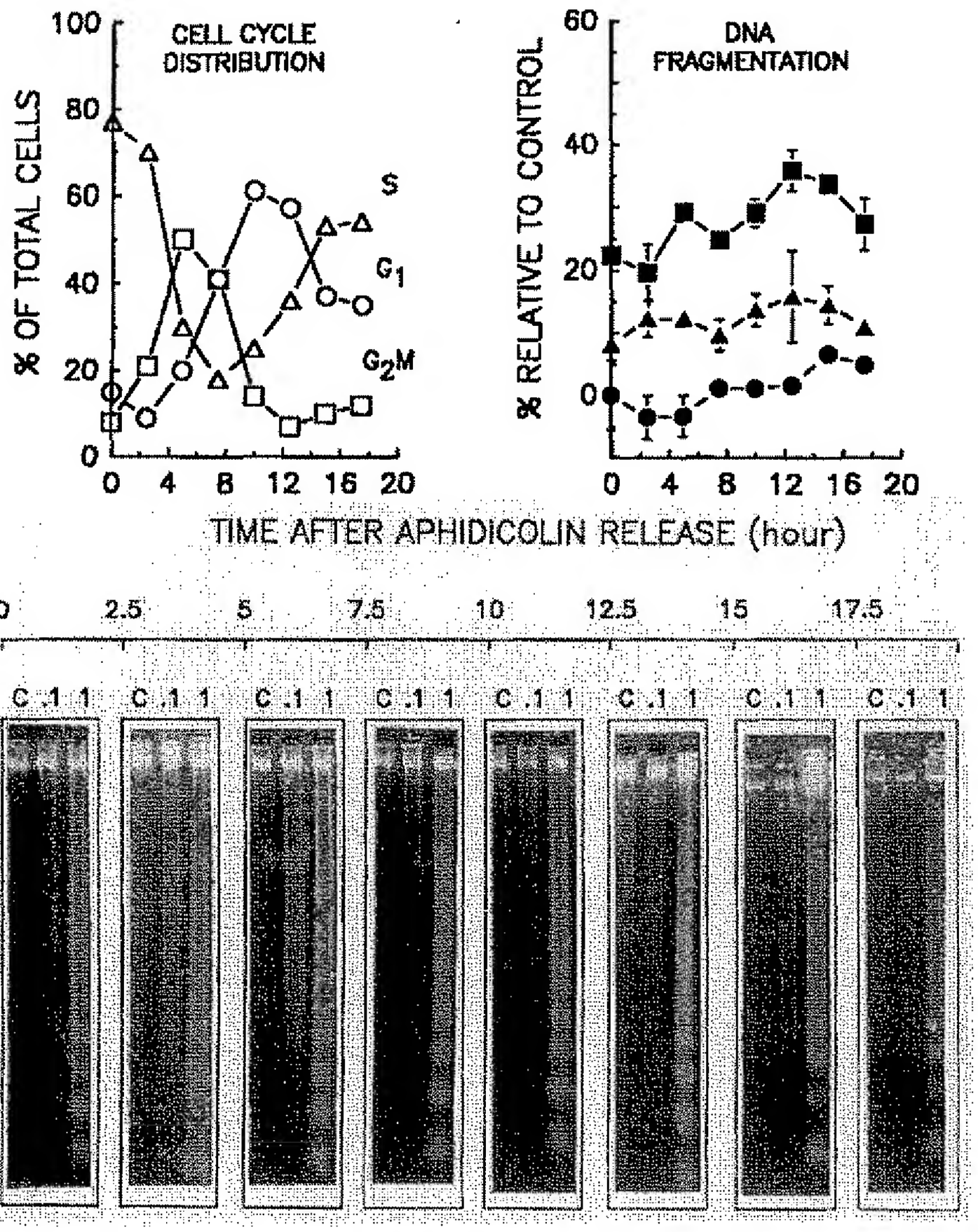

FIG. 4. Effect of cell cycle distribution upon staurosporine-induced apoptosis in CA46 cells. (Upper left panel) Cell cycle distribution after aphidicolin release (2.5-h intervals). Results are expressed as the percentage of total cells at a specific cell phase: S phase (open triangles), G1 phase (open circles), G2/M phase (open squares). (Upper right panel) DNA fragmentation was measured by filter binding assay. Synchronized cells were treated for 3 h with 0.01 μM (closed circles), 0.1 μM (closed triangles), and 1 μM (closed squares) staurosporine. The indicated times correspond to the beginning of staurosporine treatments. Points and bars shown are means ± SD of a single experiment performed in triplicate. (Lower panels) DNA fragmentation visualized by ethidium bromide-stained agarose gel. Synchronized cells were treated for 3 h in the absence (C) or presence of 0.1 μM (.1) and 1 μM (1) staurosporine. Numbers above the panels indicate the times corresponding to the beginning of staurosporine treatments.

ing staurosporine-induced apoptosis in HL-60 cells (data not shown). These results are consistent with the extraordinary potency of staurosporine to induce apoptosis.

Effect of other protein kinase or phosphatase inhibitors. Staurosporine appears to be unique among the other protein kinase or phosphatase inhibitors, since it is the only drug to induce apoptosis in all the cell lines studied (Table 1). The other inhibitors used, including two semisynthetic derivatives of staurosporine, KT-5720 and KT-5823, which are more specific c-GMP- and c-AMP-dependent kinase inhibitors [25, 26], calphostin C, a less potent but more specific protein kinase C inhibitor [27], and okadaic acid, a protein phospha-

TABLE 1

Comparative Induction of DNA Fragmentation by Staurosporine and Other Protein Kinase and Phosphatase Inhibitors

	DNA fragmentation[b]				
Cell line[a]	Staurosporine	KT-5720[c]	KT-5823[c]	Calphostin C	Okadaic acid
HL-60	96.4 ± 1.7	19.5 ± 7.2	<5	64.7 ± 18.7	77.7 ± 2.6
HL-60 (+TPA)	96.5 ± 0.7	<5	<5	<5	ND
CA46	41.8 ± 10.1	<5	<5	<5	21.6 ± 4.1
SUDHL6	24.2 ± 9.1	<5	<5	33.8 ± 13.7	10.2 ± 4.5
HT29	25.6 ± 6.3	<5	<5	<5	<5
SCL209	56.9 ± 9.8	ND	ND	<5	<5
DC3F	52.8 ± 9.8	<5	<5	<5	<5

[a] See Fig. 2.

[b] DNA fragmentation was measured by filter binding assay (see Ref. [17] and Fig. 2). Numbers represent the percentage DNA fragmentation (mean ± SD) of three independent experiments after 8-h treatments with a 1 μM drug concentration. Less than 5% DNA fragmentation was not significant at the maximal concentration tested (10 μM for KT-5720 and KT-5823, 5 μM for Calphostin C and Okadaic acid). ND, Not Determined.

[c] KT-5720 and KT-5823 are semisynthetic derivatives of staurosporine.

tase 2A and 1 inhibitor which has already been shown to induce apoptosis in some systems [28], exhibited a narrower spectrum of activity than staurosporine (Table 1).

DNA fragmentation in a reconstituted cell-free system. We were also able to elicit staurosporine-induced DNA fragmentation in a reconstituted cell-free system [19] (Fig. 5). Oligonucleosome-sized DNA fragments were induced by incubating control isolated nuclei either with the cytoplasmic fraction of staurosporine-treated cells (Fig. 5, upper panels) or with control cytoplasm incubated with staurosporine (Fig. 5, lower panels). Some DNA fragmentation was also induced by incubating control cell lysates with isolated nuclei (Fig. 5, lower panels). This may have resulted from the release of some lysosomal enzymes during the lysis procedure or slow activation of endonucleases during the incubations. Despite this background level of DNA degradation, staurosporine clearly stimulated a component within the lysate to cause significantly higher levels of DNA fragmentation than those in control lysates. Staurosporine treatment of isolated nuclei did not induce DNA fragmentation in the absence of cytoplasmic fraction (data not shown), illustrating that a staurosporine-sensitive component present in the cytoplasm stimulates the endonuclease involved in apoptosis. Such a cell-free system may prove useful in investigating apoptosis-associated endonuclease regulation. The global effect of staurosporine is consistent with experiments using high-resolution gel electrophoresis of phosphorylated proteins extracted from [^{32}P]-orthophosphoric acid-labeled cells, which showed that staurosporine rapidly and globally decreased protein phosphorylation under conditions of apoptosis induction (data not shown).

DISCUSSION

In summary, the molecular mechanisms associated with programmed cell death activation have been widely explored but not yet precisely understood. Several studies indicate that expression of some oncogenes and tumor suppressor genes could either facilitate or suppress apoptosis, depending on the stimuli used. While c-myc and wild-type p53 seem to be positive regulators of apoptosis, bcl-2 and c-Ha-ras, as well as the Epstein–Barr virus latent gene products (LMP 1), seem to be negative regulators in some instances of programmed cell death [29–37]. Mounting evidence has also pointed out pivotal roles for programmed cell death activation of growth factors, lymphokines, and cytokines which activate signal tranduction pathways involving specific tyrosine and serine/threonine protein kinase or phosphatase [38–40]. Other studies also indicate that changes in the phosphorylation state of proteins are a mandatory step during programmed cell death induced by different agents [41, 42]. Altogether, these observations and our present results with staurosporine indicate that alterations (i.e., perturbations in its/their phosphorylation state), of one or several cytoplasmic factors related to the signal transduction pathways, can trigger efficiently programmed cell death in a wide range of cell systems, regardless of their state of differentiation and cell cycle phase. Other biochemical alterations related to cell cycle [5–7] and DNA replication [43] may also contribute to the effects of staurosporine at the concentration used in the present study. These observations may be important for cancer chemotherapy since several drug research programs are aimed at developing new protein kinase and phosphatase inhibitors which may selectively kill tumor cells.

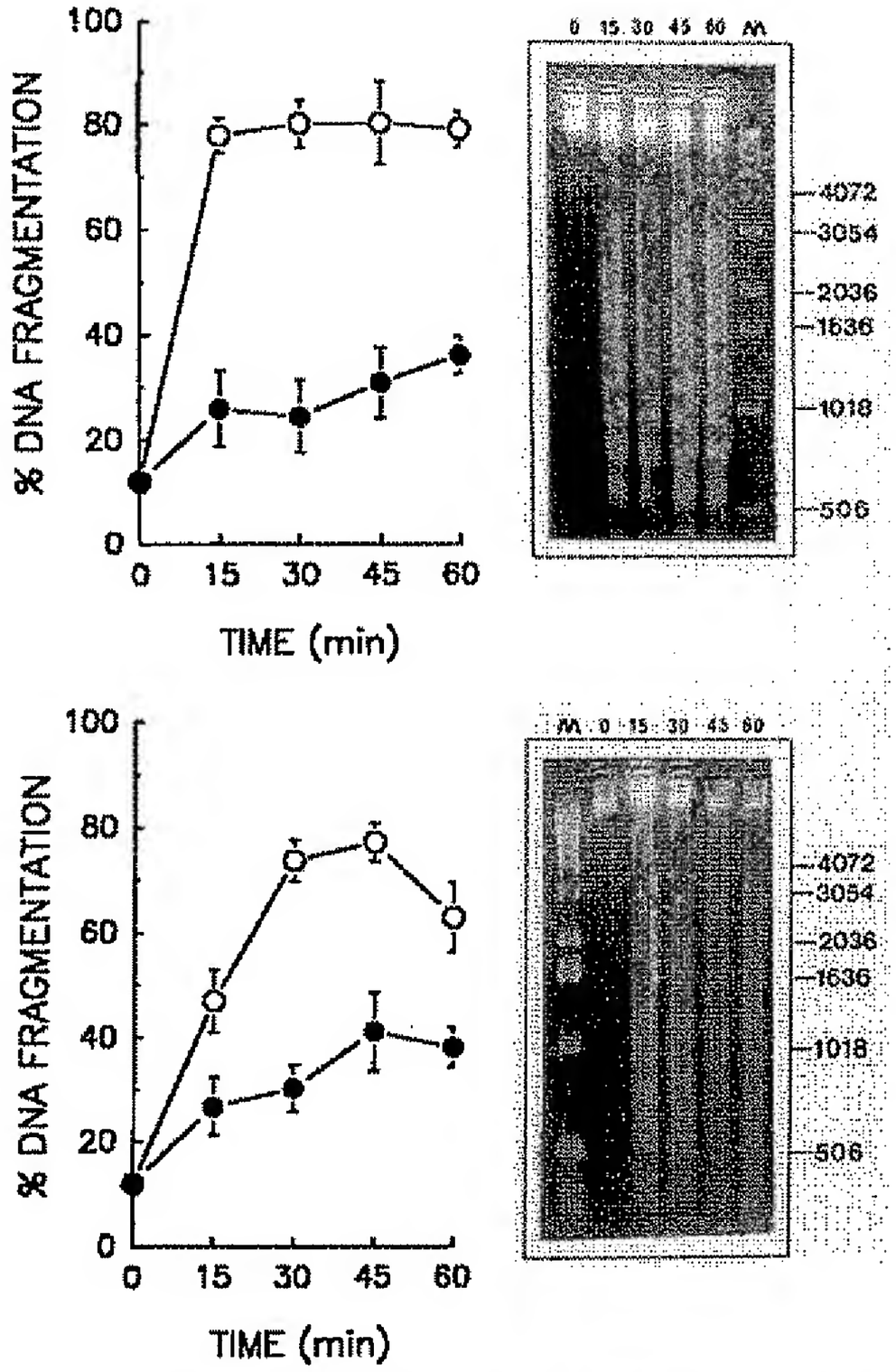

FIG. 5. Staurosporine-induced DNA fragmentation in a cell-free system. (Upper panels) DNA fragmentation induced by cytoplasmic fractions obtained from HL-60 cells treated with staurosporine (1 μM for 3 h). Cytoplasm from untreated (closed circles) or staurosporine-treated cells (open circles and upper right panel) was incubated with nuclei isolated from radiolabeled cells at 30°C for the indicated times. Results are expressed as percentage DNA fragmentation (left) and values are means ± SE of five independent experiments. The free staurosporine concentration present in the cell-free system experiments was less than 10^{-10} M. DNA fragmentation was also visualized in ethidium bromide-stained agarose gels (right). (Lower panels) DNA fragmentation induced by treatment of HL-60 cytoplasmic fraction with staurosporine (1 μM). Cytoplasm from untreated HL-60 cells was incubated with nuclei isolated from radiolabeled cells in the absence (closed circles) or presence of 1 μM staurosporine (open circles and lower right panel) at 30°C for the indicated times. Results are expressed as percentage DNA fragmentation (left) and values are means ± SE of six independent experiments. DNA fragmentation was also visualized in ethidium bromide-stained agarose gels (right).

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